

Virulence Gene Expression by *Staphylococcus epidermidis* Biofilm Cells Exposed to Antibiotics

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Staphylococcus epidermidis have become important causes of nosocomial infections, as its pathogenesis is correlated with the ability to form biofilms on polymeric surfaces. Production of poly-*N*-acetylglucosamine (PNAG) is crucial for *S. epidermidis* biofilm formation and is synthesized by the gene products of the *icaADBC* gene cluster. Production of PNAG/polysaccharide intercellular adhesin and biofilm formation are regulated by the alternative sigma factor, σ^B , and is influenced by a variety of environmental conditions including disinfectants and other antimicrobial substances. The susceptibility of five *S. epidermidis* strains to antibiotics alone and in double combination was previously tested. Our results demonstrated that some combinations are active and present a general broad spectrum against *S. epidermidis* biofilms, namely rifampicin–clindamycin and rifampicin–gentamicin. In the present study, it was investigated whether the combination of rifampicin with clindamycin and gentamicin and these antibiotics alone influence the expression of specific genes (*icaA* and *rsbU*) of *S. epidermidis* within biofilms using real-time polymerase chain reaction. The data showed that in most cases the expression of both genes tested significantly increased after exposure to antimicrobial agents alone and in combination. Besides having a similar antimicrobial effect, rifampicin combined with clindamycin and gentamicin induced a lower expression of biofilm-related genes relatively to rifampicin alone. Associated with the advantage of combinatorial therapy in avoiding the emergence of antibiotic resistance, this study demonstrated that it can also cause a lower genetic expression of *icaA* and *rsbU* genes, which are responsible for PNAG/polysaccharide intercellular adhesin production, and consequently reduce biofilm formation recidivism, relatively to rifampicin alone.

Introduction

STAPHYLOCOCCUS EPIDERMIDIS is a common commensal inhabitant of the healthy human skin and mucosa, but also an increasing nosocomial pathogen in immunocompromised patients. Living at the edge between commensalism and pathogenicity, *S. epidermidis* has developed interesting strategies to conquer the hospital environment as a novel ecological niche and to transform into a notorious pathogen.³² *S. epidermidis* causes a great number of infections, as it is the most frequent causative agent of infections of indwelling medical devices, such as peripheral or central intravenous catheters.²⁹ These infections usually commence with the introduction of bacteria from the skin of the patient or that of healthcare personnel.^{1,25} The increasing use of invasive devices for diagnostics and therapeutic procedures has contributed to an increase in the number of *S. epidermidis* medical device-related infections, ranking this bacterium first among the causative agents of nosocomial infections.

The tight pathogenic association is essentially linked to the species' ability to form adherent biofilms on artificial surfaces,³⁰ which is the main virulence mechanism of *S. epidermidis*. Biofilm-associated bacteria are 100–1,000 times less susceptible to antibiotics than are planktonic cells.⁶ A number of factors have been postulated as mechanisms of biofilm-associated antibiotic resistance, including binding of antibiotics to the slime, poor penetration of antibiotic into the biofilm, slow growth rate of organisms in the biofilm, high bacterial density, and change in gene expression in biofilm bacteria.^{6,33}

Antibiotic combinations are being increasingly used as therapeutic option as a result of the increasing appearance of multiresistant microorganisms.²¹

Many *S. epidermidis* strains produce poly-*N*-acetylglucosamine (PNAG), also named polysaccharide intercellular adhesin (PIA) homopolymer, a crucial factor for biofilm formation, which surrounds and connects *S. epidermidis* cells in a biofilm.²⁰ Synthesis of PIA is essential for bacterial cell

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accumulation because it mediates cell-to-cell adhesion of proliferating cells.¹² In addition to its role as part of the extracellular biofilm matrix, PNAG/PIA has been found to protect the bacterium from important mechanisms of innate host defense, namely neutrophil killing, complement deposition, immunoglobulins, and antimicrobial peptides.^{14,35} The correlation of the presence of PNAG/PIA with the invasiveness of the bacterium may be due to the roles of this exopolymer in biofilm formation and immune evasion.²⁶ Two main putative determinants of *S. epidermidis* invasiveness have been identified: the *ica* genes, which regulate the production of PNAG/PIA, and the insertion element *IS256*. The latter is thought to contribute to the genetic adaptation that may have a role during infection.³⁶ The biosynthesis of PNAG/PIA is accomplished by the gene products of the *ica* (intercellular adhesion) locus,^{8,10,20} which comprises four intercellular adhesion genes *icaA*, *icaB*, *icaC*, and *icaD*.²³ The expression of the *ica* operon and biofilm formation depends on a variety of environmental conditions. The expression of at least four unlinked genes (*icaR*,⁷ σ^B , the *purR* locus, and *sarA*) is known to control PNAG/PIA synthesis at the level of transcription.^{3,18,19} Production of PNAG/PIA is subject to a range of regulatory influences,²⁴ including many global virulence regulators. PNAG/PIA and biofilm formation is regulated by the alternative sigma factor σ^B , which is presumed to play a crucial role in the global regulation of gene expression.¹¹ *RsbU* is a positive regulator of the activity of σ^B , the general stress-response factor of Gram-positive microorganisms.⁵ σ^B may act only indirectly via an additional, unknown factor or *rsbU* may, by itself, be a regulator of *icaADBC* transcription.¹²

Activation of PNAG/PIA expression by different stress stimuli apparently uses different pathways. This implies that a number of environmental conditions and regulatory systems can influence the expression of staphylococcal biofilms, reflecting the magnitude of the complexity associated with biofilm formation.²⁷

In previous studies, we assessed the effect of various antibiotics alone and in double combination against *S. epidermidis* biofilms. Rifampicin combined with gentamicin and clindamycin showed to be the most active combinations, promoting reductions of about 3 log colony forming units (CFUs) in biofilm cells in the majority of clinical isolates tested. However, the effect of these treatments in the subsequent response of survival cells was never determined. Accordingly, the main goal of the present work was to assess the expression of some genes of *S. epidermidis*, namely *icaA* and *rsbU*, both responsible by PNAG/PIA production after antibiotic treatment.

Materials and Methods

Bacterial strains and antimicrobial agents

In this study, five good-biofilm-producing and *ica*-positive *S. epidermidis* strains were used: 117977, 132034, 150271, 1457, and 9142. The first three strains were kindly provided by Dr. Howard Ceri, Department of Biological Sciences, University of Calgary, Calgary, and the last two by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston. These strains are clinical isolates (isolated from infected catheters) and were stored at -80°C . Anti-

biotics tested were rifampicin, gentamicin, and clindamycin (Sigma) and were used at break-point concentration. Interpretation criteria for susceptibility testing were based on National Committee for Clinical Laboratory Standards,²² now Clinical Laboratory Standards Institute (CLSI) guidelines (Table 1).

Biofilm formation

Several colonies of the isolates grown on tryptic soy agar plates were suspended in saline (0.9% NaCl) to a density of 1.0 on the McFarland scale, as indicated by the manufacturer. Then the bacterial suspension was resuspended in tryptic soy broth to obtain a cellular concentration of *circa* 1×10^7 CFU/ml. This solution was used as inoculum for the MBEC™ device (MBEC Biofilm Technologies Ltd.). The biofilms were grown for 48 hours at 37°C and 150 rpm on a rocking platform where the shear force was created against the pegs forming 96 equivalent biofilms. At the end of the incubation period, a biofilm of approximately 6 logs per peg was formed.

Biofilm challenge and recovery

The challenge plates were prepared using the antibiotics at break-point concentration (Table 1) alone and in combination (rifampicin + clindamycin and rifampicin + gentamicin). The biofilms formed on the lid of the MBEC were rinsed twice with 0.9% saline and placed into the challenge plate overnight at 37°C , 150 rpm on a rocking platform, and 95% relative humidity. Then, the challenged biofilms were rinsed twice in saline and transferred to a recovery plate that consisted of tryptic soy broth medium plus 1% Tween. Biofilms were removed from all pegs at once by sonication for 8 minutes on high with an Aquasonic sonicator (model 250HT; VWR Scientific).² The vibration disrupted biofilms from the surface of the 96 pegs into the recovery plate. Then, CFUs were determined as follows: the recovery medium (containing the sonicated biofilms) was serially diluted. The biofilm cultures (10-fold diluted) were spotted on tryptic soy agar plates. Then the plates were incubated for 48 hours at 37°C to ensure maximum recovery of the surviving microorganisms and CFUs were counted. The same protocol was repeated and all samples were collected, preserved in RNAlater® solution (Ambion-Applied Biosystems), and kept at -80°C for further study of gene expression.

Genetic expression analysis

Primer design. Primers used for *S. epidermidis* *icaA* and *rsbU* genes analysis by real-time polymerase chain reaction (PCR) were designed using Primer3 web-based software

TABLE 1. ANTIBIOTICS' BREAK POINTS

Antibiotic	Break point ($\mu\text{g/ml}$)			Reference ²²
	Sensitive	Intermediate	Resistant ^a	
Rifampicin	1	2	4	NCCLS
Gentamicin	4	8	16	NCCLS
Clindamycin	0.5	—	2	NCCLS

^aConcentration used in bactericidal antibiotic test.

NCCLS, National Committee for Clinical Laboratory Standards.

(http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)³¹ and are listed in Table 2. To verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from 1457 strain genomic DNA (data not shown).

RNA extraction. Total RNA of each sample was extracted using the PureLink™ RNA Mini Kit (Invitrogen) according to the manufacturer's recommended protocol. Potential DNA contamination was removed during RNA purification procedure by On-column PureLink DNase treatment (Invitrogen). RNA concentration (ng/μl) and purity (OD_{260nm}/OD_{280nm}) were assessed by spectrophotometric measurements using a NanoDrop device (NanoDrop 1000 Spectrophotometer, V3.6.0; Thermo Fisher Scientific, Inc.).

cDNA synthesis. To ensure equivalent starting amounts of RNA from control and respective treated samples to be converted into cDNA, appropriate dilutions in RNase-free water were performed to obtain a final concentration of 2 μg of RNA. cDNA of each sample was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.). Each reaction contained 2.5 μl of iScript Reaction Mix + iScript Reverse Transcriptase and 7.5 μl of RNA template, in accordance with the proportions recommended by the kit manufacturer, making a final reaction volume of 10 μl. Complete reaction mix was incubated in a thermocycler (MyCycler; BioRad) with the following reaction protocol: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C.

Quantitative real-time PCR. Real-time PCRs were performed on a CFX96™ real-time PCR Detection System (Bio-Rad Laboratories, Inc.). Each 20 μl of reaction mixture contained 2 μl of cDNA, 1 μl of each primer (forward and reverse primers), 10 μl of 1× SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), and 6 μl of nuclease-free water. Thermal cycling conditions were as follows: 3 minutes initial denaturation at 95°C, followed by 40 cycles of 5 seconds denaturation at 95°C, 10 seconds annealing/extension at 60°C (this step was performed at 60°C, concerning primers efficiency previously determined [data not shown]). A melt curve was inserted at the end of each run, with readings from 65°C to 95°C, every 1°C for 5 seconds, to confirm that only the desired product was amplified.

Gene analysis and expression. Samples for real-time PCRs were run in triplicate. Data were analyzed using the CFX Manager™ version 1.6 (Bio-Rad Laboratories, Inc.) and

the relative quantification method ($2^{-\Delta\Delta CT}$; Livak and Schmittgen),¹⁷ which describes the change in expression of the target genes relative to the 16S rRNA reference genes from untreated control samples,^{15,34} and the cycle threshold values (CT; cycle at which each sample amplification curve crosses a specific threshold) were averaged for triplicate samples. The ΔCT values of the target genes were determined by normalizing to the endogenous control genes 16S rRNA. These samples were subsequently subtracted from the 16S rRNA genes of the untreated control samples. The $\Delta\Delta CT$ was used to calculate relative expression using the following formula: $2^{-\Delta\Delta CT}$.^{9,16,17}

The cDNA was validated by comparing with an RNA sample not subjected to reverse transcriptase reaction, to determine possible DNA contamination. All these no-reverse transcriptase controls showed a $\Delta\Delta CT$ above 10 cycles, indicating that the cDNA present resulted from the conversion of the RNA.

Statistical analysis

The data were analyzed by applying two-tailed independent samples *t* tests. SPSS software was used to perform the analysis. All tests were performed with a confidence level of 95%.

Results

We have previously demonstrated that some combinations of antibiotics are significantly effective against *S. epidermidis* biofilm cells. Rifampicin alone was generally as or more effective than when used in combination with other antibiotics (Fig. 1). However, this kind of combinatorial therapy aims to prevent the emergence of resistance usually associated with monotherapy. Two of the most efficient combinations that induced a greater reduction of bacterial biofilm population and demonstrated a broader spectrum range, being active against all strains tested, were rifampicin plus clindamycin or gentamicin. Both these combinations

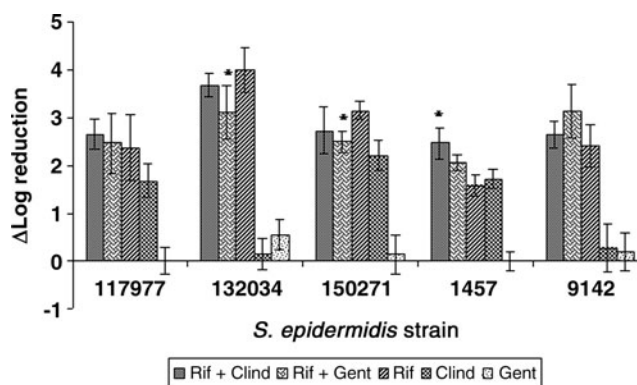


FIG. 1. Effect of rifampicin (Rif), clindamycin (Clind), and gentamicin (Gent) alone and in combination (Rif + Clind; Rif + Gent) on 48-hour-old biofilm cells of five *Staphylococcus epidermidis* clinical isolates, after overnight exposure. Δ Log reduction = differences between positive control (untreated control: 6 log₁₀ colony forming units/peg) and treated samples in log₁₀ colony forming units/peg. Error bars represent standard deviation. *Combinations statistically different from rifampicin alone ($p < 0.05$).

TABLE 2. LIST OF THE PRIMERS USED FOR THE REAL TIME-POLYMERASE CHAIN REACTION EXPERIMENTS

Primer name	Sequence	Target
16SRNAFW	gggctacacacgtgctacaa	16S
16SRNAREV	gtacaagaccgggaacgta	16S
rsbUFW	taacgtgttttgggactcacac	usbU
rsbUREV	tgttgaaaagaacgttaacaaa	rsbU
icaAFW	gcactcaatgaggaatca	icaA
icaAREV	taactgcgcctaatttggatt	icaA

were able to reduce bacterial cell number by approximately 3 log (Fig. 1) for all strains tested.

To determine the effect of rifampicin, clindamycin and gentamicin, and rifampicin combined with clindamycin or gentamicin on gene expression of *S. epidermidis* *icaA* and *rsbU* genes, real-time PCR analysis was performed. The results obtained for the different strains tested are represented in Fig. 2.

Relative to the unexposed control, *icaA* and *rsbU* were overexpressed in all strains when exposed to rifampicin at break-point concentration ($p < 0.05$), except for strain 132034 (Fig. 2B). The same was observed for gentamicin, which caused the overexpression of both genes in four strains ($p < 0.05$), with the exception of 1457 *S. epidermidis*. Generally,

no overexpression of *icaA* and *rsbU* genes was observed in biofilm cells exposed to clindamycin. Relatively to combinations of antibiotics, the response of *S. epidermidis* was strain dependent. Three strains demonstrated overexpression of both genes after exposure to rifampicin + clindamycin and rifampicin + gentamicin when compared with untreated biofilms ($p < 0.05$). On the contrary, these two combinations induced subexpression of *icaA* and *rsbU* on 1457 *S. epidermidis*. On the other hand, in strain 150271, both combinations provoked subexpression of *icaA* and the expression of *rsbU* was unaffected comparatively to the positive control ($p < 0.05$).

Comparing the effect of antibiotics alone and in combination, it was observed that the combinations always

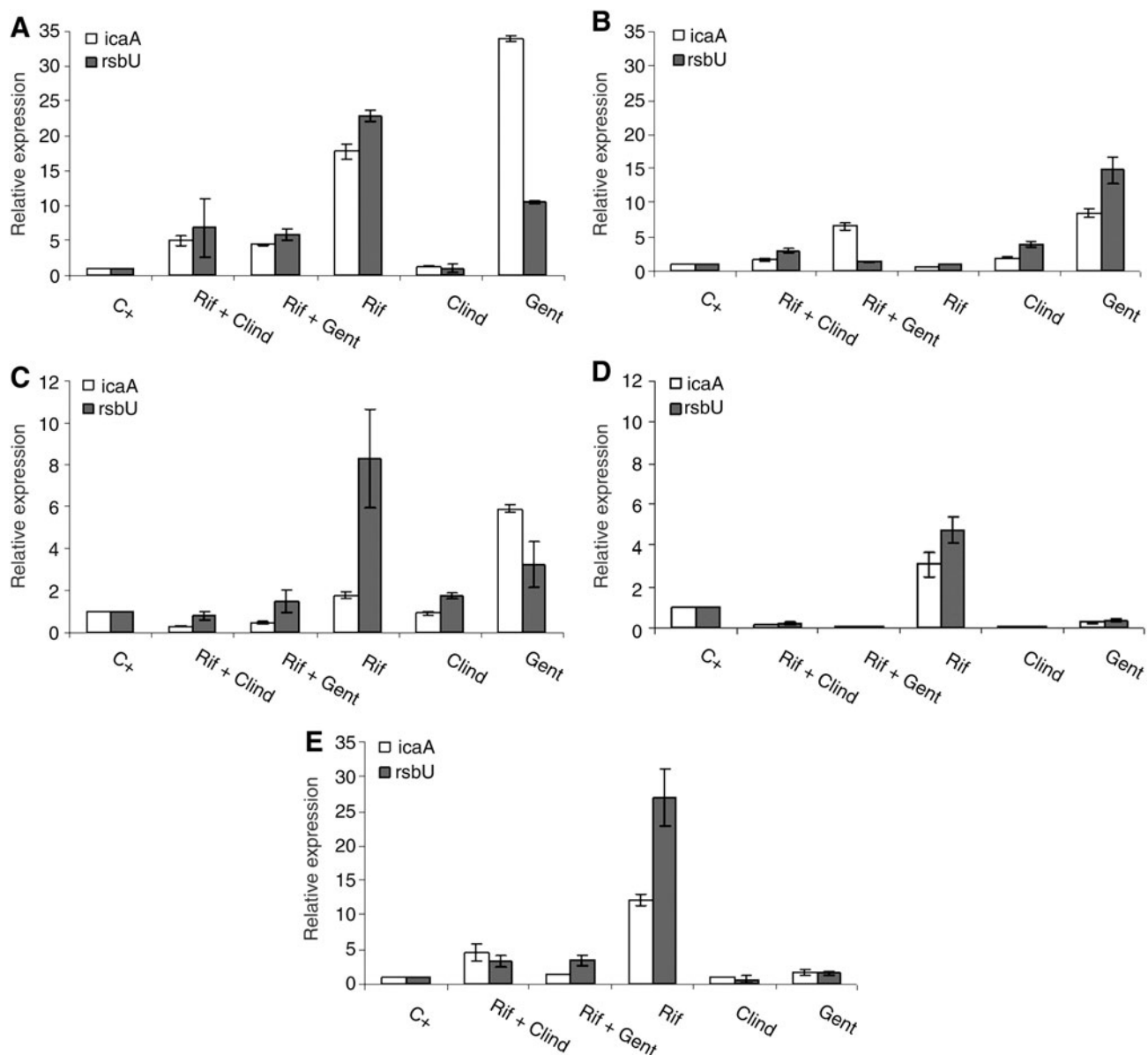


FIG. 2. Expression of *icaA* and *rsbU* in *S. epidermidis* strains (A) 117977, (B) 132034, (C) 150271, (D) 1457, and (E) 9142, in response to rifampicin (Rif), clindamycin (Clind), gentamicin (Gent), rifampicin combined with clindamycin (Rif + Clind), and rifampicin with gentamicin (Rif + Gent). The relative expression of *icaA* and *rsbU* in biofilms exposed to antibiotics was plotted against unexposed control biofilms (C+), using *16S* as the reference gene. The $2^{-\Delta\Delta CT}$ was calculated from the average cycle threshold (CT) values of two reactions. Error bars represent standard deviation.

induced expression of *icaA* and *rsbU* genes in a lesser extent than antibiotics individually. As clindamycin and gentamicin are not active antimicrobial agents against *S. epidermidis* biofilms (Fig. 1), and rifampicin was the only antibiotic presenting similar effect to the most active combinations tested (ca. 3 log reduction), it seems important to compare the genetic expression of both genes after biofilm exposure to these antimicrobial treatments. Therefore, although both combinations of antibiotics induce an increase of *icaA* and *rsbU* expression in response to an environmental stress, this increase is not as marked as in cells exposed to rifampicin alone. In fact, comparing the *icaA* and *rsbU* gene expression after treatment with rifampicin and rifampicin combined with clindamycin or gentamicin, we can conclude that, with the exception of 132034, there is always a general decrease of the expression of these two genes after biofilm exposure to both combinations ($p < 0.05$) (Fig. 2).

Discussion

Our results showed that normally the expression of *icaA* is accompanied by the expression of *rsbU*. Pintens *et al.* demonstrated that the increase in *icaA* expression clearly preceded the increase in *rsbU* expression *in vitro* as well as *in vivo*.²⁷ This is consistent with factors other than *sigB* inducing *icaADBC* operon expression.^{3,18,19}

RsbU is a positive regulator of the alternative sigma factor σ^B , which is known to control PNAG/PIA synthesis at the transcription level. Moreover, the global stress response regulator σ^B and *rsbU* control biofilm development in *S. epidermidis*.¹² The results clearly indicated that the expression of *icaA* and *rsbU* is generally inferior in the presence of breakpoint concentrations of rifampicin combined with clindamycin or gentamicin in comparison to rifampicin alone. Although all treatments studied induced the expression of the two genes assayed, after overnight contact, rifampicin is a stronger inducer of *icaA* and *rsbU* genes expression, possibly inducing in *S. epidermidis* persister cells a high ability for biofilm formation. In fact, biofilm formation has been reported to be induced by conditions that are potentially toxic for bacterial cells, such as high levels of osmolarity, detergents, urea, ethanol, oxidative stress, and the presence of sub-MICs of some antibiotics such as tetracycline and quinupristin-dalfopristin.^{4,13,28} In the present study, it was verified that a toxic condition, caused by rifampicin exposure, promoted an increased expression of *icaA* gene by surviving cells. In *S. epidermidis*, the induction of PNAG/PIA production and biofilm formation can improve the ability of biofilm immune evasion. PNAG/PIA is crucial for connection of cells in a biofilm, avoiding the detachment of biofilm cells, mechanism that can make the cells more susceptible to antimicrobial agents. Further, this exopolymer has also a protective function. Consequently, the induction of PNAG/PIA formation can be a possible defense and resistance mechanism of cells.

Therefore, in addition to the already known advantages of antibiotic combinatorial therapy, for example, as a strategy to reduce resistance development, our results show that the use of rifampicin combined with gentamicin and clindamycin may be a better therapy strategy and more effective than rifampicin alone (the most efficient antibiotic against *S. epidermidis* used in clinical practice). The combination therapy, in addition to allowing for an almost equally ef-

fective killing of bacterial cells, results in lower expression of *icaA* and *rsbU*, and consequently less PIA production, than if rifampicin was used alone, thereby ensuring a more efficient control of *S. epidermidis* biofilm-associated infections.

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Disclosure Statement

No competing financial interests exist.

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